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Heterotrophic bacterial production measured on soil microaggregates sampled using a Biological Laser Printer

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ABSTRACT

Although routinely measured in aquatic systems over the last 30 years, heterotrophic bacterial production (^{3}H-leucine method) has only more recently been measured in terrestrial ecosystems to elucidate the role of soil biogeochemical processes in global carbon cycles and climate change. Studying bacterial metabolism at the microaggregate scale (100–300 μm) may unmask important microscale biogeochemical relationships amongst bacterial processes and the surrounding soil. Biological laser printer technology was used to sample soil core sections (*ca.* one mm thick) into *ca.* 100–300 μm microaggregates. Bacterial production was measured on collections of five microaggregates each (180 samples over 5 cm depth profile) by suspending microaggregates into filtered rainwater with ^{3}H-leucine and incubating for 4 h. Bacterial production varied by over two orders of magnitude amongst groups of five microaggregates collected over the same millimeter scale core slice. Range for all measurements along the core was below detection (0.005) to 897 fg C aggregate d^{-1}. As expected, variability amongst adjacent (within 2 mm of each other) microaggregates groups generally decreased with depth along with the total magnitude of bacterial production rate. Variation in bacterial production at the microaggregate scale was greater than that seen at larger scale (*ca.* 50 mg; theoretical 3200 microaggregates) sampling of the same core. This is the first demonstration of using a biological laser printer for fine scale sampling of soil core slices and subsequent measurement of bacterial metabolism.

1. Introduction

The 100–300 μm microaggregate may be the functional unit of soil that is most important to understanding organic matter cycling and preservation (Six et al., 2004; Vos et al., 2013, and references therein). The objective of this work was to investigate the utility of a Biological Laser Printer (BioLP) for subsampling soil at this scale. BioLP involves using a laser pulse striking a film on a quartz plate to nondestructively dislodge a soil aggregate or liquid bead from the other side of the plate into a collection vessel (*e.g.,* microtiter dish, microfuge tube) (Ringeisen et al., 2015). Sampling size can be dictated by energy density and width of the laser beam (Barron et al., 2004). Once on the quartz plate, media is sampled at high resolution and high throughput while maintaining the original spatial coordinates of each laser-generated subsample relative to other adjacent subsamples. This allows for nearest neighbor analyses of chemical or biological properties that can be measured at that sample size (Ringeisen et al., 2015). Thus far, BioLP has been used for high resolution sampling of eukaryotic tissue (Wu and Ringeisen, 2010), viruses (Fitzgerald et al., 2010), liquid broth, seawater, isolation of electrochemically active microorganisms, and sediment supernatant (Ringeisen et al., 2010). Other 3D printing strategies have been used to study macropore networks and porosity in soil (Bacher et al., 2015; Jassogne et al., 2007) but not as a method to sample soil microaggregates (Ringeisen et al., 2015).

There has been recent interest in examining the importance of microscale processes to both marine and terrestrial biogeochemical cycles (Stocker, 2015). Historically, soil science has maintained a large focus on fostering plant biomass production in agriculture (Baveye, 2015) but

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emerging understandings of the importance of soil microarchitecture (Six et al., 2004) and recent interest in the role of soil in carbon sequestration and climate change has piqued interest in the relationship between soil processes and global biogeochemical cycles (Six and Paustian, 2014; Crowther et al., 2016; Bond-Lamberty et al., 2018).

Measures of heterotrophic bacterial production are typically employed to study carbon cycling in marine ecosystems (*e.g.,* Giering et al., 2014). The leucine method (Kirchman, 2001) measures rates of protein synthesis and is commonly used in marine ecology but has been used to a lesser extent in soil ecology (*e.g.,* Bååth et al., 2001). One reason may be that although this method is relatively convenient and straightforward to use with aquatic samples, presence of soil or sediment can quench the light produced in scintillation fluid used in this radioassay and decrease the assay's sensitivity (*e.g.,* Cook et al., 2007). One strategy for dealing with this limitation is to add steps to separate extracted bacterial protein from the sample matrix to eliminate quench but this introduces extra time and an extraction efficiency error that can be cumbersome to quantify and may vary with bacterial assemblage type and different chemical environments (Bååth et al., 2001; Findlay et al., 1984; Marxsen, 1996). A second strategy is to reduce the sample size to ameliorate quench (Montgomery et al., 2010) but this does not totally eliminate particles and also the amount of quench may also still vary with different chemical environments. Using a BioLP to sample soil and sediment at the microaggregate scale may eliminate the need to separate proteins from soil as the tiny mass of particles would have a minimal effect on radioassay quench. More significantly, BioLP sampling would allow measurement of bacterial growth at the likely scale of heterogeneity of cell abundance and nutrient conditions within soil structure (Pedersen et al., 2015). Currently, these correlations are made using macroscale measurements in soil (*e.g.,* grams, cm^3) but this strategy can average out important individual features in the 3D substructure of a soil column with depth (Lehmann et al., 2008). Important correlations among co-varying rates will be lost when the sampling scale is larger than the microscale gradients upon which these processes change (Falconer et al., 2015; Gupta and Germida, 2015).

2. Material and methods

2.1. Media collection

Soil was collected with a core liner tube (Wilco; #3–2447-C41) bored into the surface of a residential yard and operational corn field at Frying Pan Park; both in Fairfax County, VA. Rainwater was collected in a stainless-steel bowl and filtered (0.2 μm nom. pore dia., Nalgene cellulose acetate #190–2520) immediately after collection and again prior to use and stored at 4 °C.

2.2. BioLP soil sampling

BioLP apparatus has been previously described (Barron et al., 2004, 2005; Ringeisen et al., 2010) and has been used for soil supernatant sampling (Ringeisen et al., 2015), however, in this study, microaggregates were collected into 1.8 mL microfuge tubes (Fisher #02-681-344 with O-ring #02-681-363) rather than into a microtiter well plate. This adaptation allowed for the subsequent measurement of bacterial production without transfer losses from collection to assay. Briefly, the BioLP process requires a transparent quartz slide to be coated with an ultraviolet (UV) absorption coating. In these experiments, an 85 nm thick titanium dioxide coating was used on a 3.175 mm thick 50.8 mm diameter, square quartz slide. The UV absorptive coating is used to initiate the printing process as the UV laser pulse absorbs at the titanium/quartz interface inducing an energetic expulsion of material ("ink") that is typically layered above the titanium dioxide coating. For these experiments, that "ink" was an approximately 0.3175 cm thick 5.04 cm diameter slice of soil spread out to be *ca.* 300 μm thick prior to sampling.

To generate these soil subsections for printing, soil cores were subcored with a 3 mL plastic syringe with the tip cut off leaving a 1 cm ID. The core tub was pressed into the larger core 3.5 cm to create a sample that was at least 3 cm deep and 1 cm in diameter. This core was extruded 1 mm at a time and sliced with a fresh and sterile single sided razor blade producing a 1 mm thick disk 10 mm in diameter. These disks were further sectioned using a custom stainless-steel die (Apple Die, Milwaukee, WI; Figure S1 supplementary data). The die has 9 equal-sized 2 mm × 2 mm squares in the center, around the perimeter, each division is at least 4 mm^2. The die was washed and sonicated in 70% ethanol after each dissection.

Prior to inversion and sampling with the laser, a drop of Milli-Q water (approx. 50 μL) was added to the soil slice prior to flattening onto the quartz slide to help adhere the soil to the UV absorptive coating though filtered rainwater would be a better choice. Laser pulses of 266 nm wavelength, 3 ns pulse width, 200 μm diameter, and 50 μJ were generated at close to 10 hz by an RPMC Lasers WEDGE XB single shot laser (Model#: XB266). Pulses were actually triggered to "in position" cues for the microfuge tubes held in the receiving substrate. Each pulse would transfer microaggregates of soil from the quartz support to a 1.8 mL microfuge tube. Abundance of aggregates per tube can be controlled via computer aided stages and control of the pulsed laser.

2.3. Heterotrophic bacterial production

Bacterial production was determined using the leucine method (Smith and Azam, 1992) with conversions and sampling as described by Montgomery et al. (2010) for the macroscale soil assay (*ca.* 50 μL via cut-off 1 mL syringe, here equaling 54–63 mg dry weight of soil). However, for the BioLP sampled soil, L-[2,3,4,5-3H]-leucine (^{3}H-Leu; spec. act.: 100–150 Ci $mmol^{-1}$, American Radiochemicals Inc., St. Louis, MO; 20 nM final concn) was first mixed with filtered rainwater (300 μL) which was added to each microfuge tube rather than having tubes precharged with ^{3}H-leucine prior to adding the microaggregates. All microaggregate treatments were incubated for 4 h rather than the typical 0.5–1 h because of the expected bacterial abundance per microfuge tube. For both macroscale and BioLP soil sampling, incubations were performed at RT (*ca.* 26 °C), then terminated with trichloroacetic acid (TCA; 5% final concn) and stored frozen (−4 °C) prior to processing. Briefly, processing involves microcentrifugation of the precipitated bacterial protein, aspiration of supernatant and rinsing of pellet with 5% TCA, then 80% ethanol prior to addition of scintillation cocktail and radioassay.

An estimate of 64,000 microaggregates per cm^3 (each aggregate with theoretical dimensions *ca.* 250 μm × 250 μm x 250 μm) and the dry weight (theoretically 85–98 μg for 5 microaggregates) were used to convert production from $microaggregate^{-1}$ to g^{-1} scale derived from the macroscale soil assay method (Montgomery et al., 2010). Values for zero-time controls (TCA added at start of incubation) were subtracted from those for live sample prior to rate measurement calculations. Assay detection limit (DL) was 0.005 fg C produced by bacteria per aggregate per day. Error bars for the macroscale assay were the SD of triplicates minus the zero-time control. Given the microaggregate scale of heterogeneity reported for soil systems (*e.g.,* Falconer et al., 2015), adjacent groups would be pseudoreplicates so these values were presented as individual observations (live minus zero-time control) and not averaged in Figs. 2 and 3. To determine the amount of microaggregates needed to detect the amount of bacterial production, results are presented in terms of amount of radioactivity (DPM of ^{3}H-leucine into bacterial proteins) rather than converting into bacterial production.

For the macroscale core sampling, bacterial production was measured with depth in five areas along a 30-cm soil core taken from a residential yard. Core subsampling involved the A and B horizons with a focus at their interface. A one-mL plastic syringe with the end cut off was used to sub-core along the depth of the original soil core by extracting the plunger as the syringe barrel was inserted into the side of

the core. The value for each of five depths represents a single collection of live aggregates minus a single zero-time control for the same number of aggregates. For the microscale sampling, BioLP was used to subsample *ca.* 100–300 µm microaggregates from soil core slices from a corn field and a residential yard. A 30 cm deep core was taken from the surface of an operational corn field and sliced (*ca.* 1 mm thick), subsectioned and sampled down to 5 cm deep. Rate of heterotrophic bacterial production was measured on groups of five adjacent microaggregates at one mm depth increments from 0 to 1.2 cm and then ca. every 2 mm from 1.4 to 5.0 cm. Each value (n = 114) represents a collection of 5 microaggregates (live value minus zero-time control blank for that depth) collected sequentially within a 1 mm × 2 mm x 2 mm subsection at a given depth. Values from each depth are from the same slide.

Two triplicate samples were also collected from the same corn field core for the macroscale soil assay at depths of 1 and 5 cm and compared with same depth samples processed with the BioLP (and subsequently normalized to dry weight (g) using the hypothetical 64,000 microaggregates per cm^3 of soil).

3. Results

3.1. Macroscale core sampling

Bacterial production generally decreased with depth from 784 (± 197) µg C kg^{-1} soil d^{-1} at 5.4 cm below the surface to 142 (± 31) to 222 (± 36) µg C kg^{-1} soil d^{-1} in the B horizon (14.6 cm below surface; Fig. 1) as has been seen elsewhere (*e.g.*, Blume et al., 2002). Of note is that the sample closest to the A-B horizon interface (13.5 cm deep) had the highest coefficient of variation at 46% of the mean bacterial production for that depth, whereas coefficient of variation for all other samples ranged 16–25%. This may be due to the scale of heterogeneity present at this interface between the two horizons.

3.2. Detection limit of bacterial production on microaggregates

DPM (=Disintegrations Per Minute) associated with zero-time controls from the corn field ranged from a low of 272 DPM at three microaggregates to a high of 852 DPM at 20 microaggregates (Fig. 2). Mean value for all zero-time controls from residential and corn field soil was 543 (± 162) DPM. Though the zero-time value did slightly increase with increasing microaggregate abundance, it appears that much

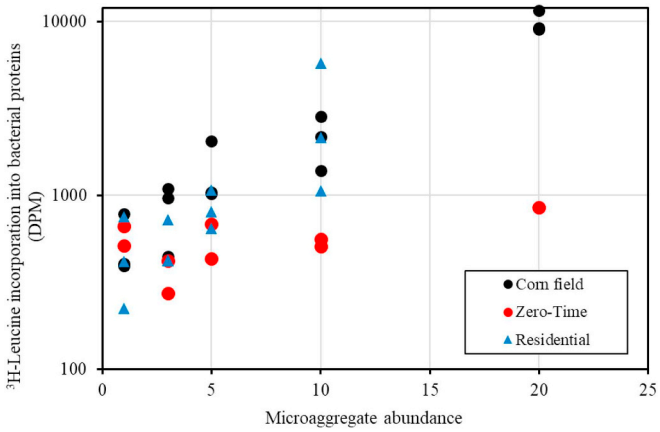

Fig. 2. Amount of ^{3}H-Leu incorporated into bacterial protein by radioassay (DPM; Disintegrations Per Minute) as a function of abundance of microaggregates added to each sample from a corn field (black circles) and residential yard (blue triangles) relative to zero-time controls (red circles) during a 4-h incubation. Plotted are 3 live values and a zero-time control for each soil sample (n = 9). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

of the background DPM may be associated with unincorporated ^{3}H-Leu binding to the microfuge tube walls. Bacterial production values for live samples using groups of one or three microaggregates were indistinguishable from that for the zero-time samples. Although this mean value for the zero-time control (543 DPM) is low relative to the *ca.* 2220000 DPM added to the original incubation, it may limit the assay to using groups of five microaggregates at least for soils with this level of microbial activity and incubation time (Fig. 2). For 15 observations of 5 or more microaggregates, there was a mean value of 219 DPM aggregate^{-1} incorporated with a range of 20–551 DPM (Fig. 2).

3.3. Bacterial production of microaggregates with depth

Measurable rates (above the zero-time control; Detection Limit, 0.005 fg C aggregate^{-1} d^{-1}) were observed in 23 of 114 values and ranged from 0.05 to 897 fg C aggregate d^{-1} (Fig. 3). About two orders of magnitude range in rates was measured among adjacent microaggregate groups (*e.g.*, 2.8–897 fg microaggregate^{-1} d^{-1} within the

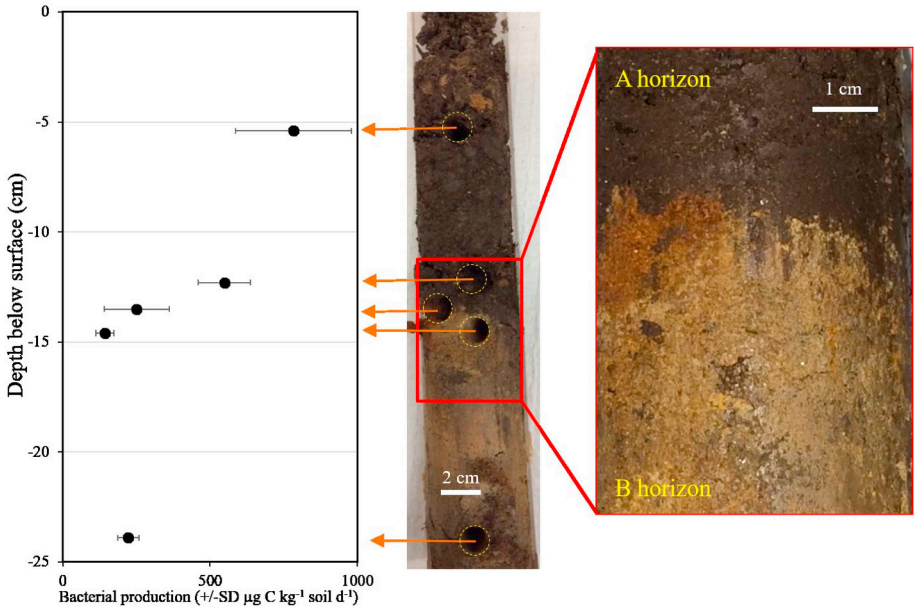

Fig. 1. Heterotrophic bacterial production (+/− SD µg C kg^{-1} soil d^{-1}) measured with depth along a residential soil core (center) with sampling focused at the interface between the A and B horizons (right blowup, prior to syringe sampling). Syringe sample locations are highlighted (yellow dashed line). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

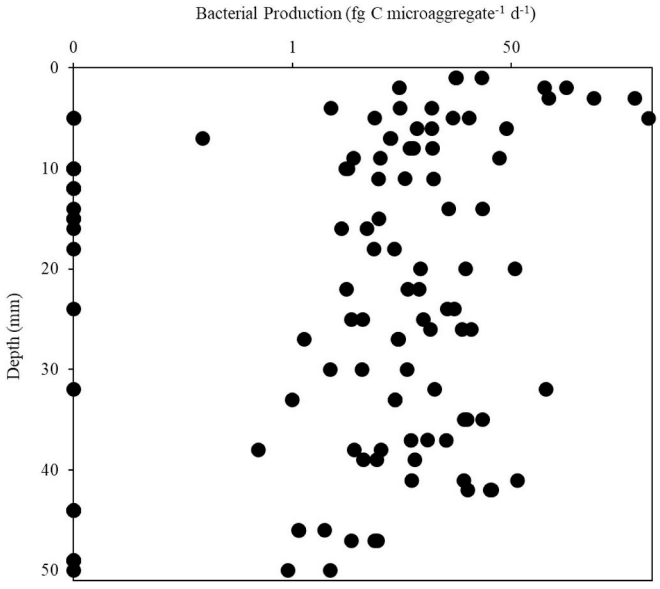

Fig. 3. Bacterial production (fg C microaggregate^{-1} d^{-1}) of groups of five microaggregates sampled using the BioLP with depth (mm) below the surface of a soil core from a corn field (23 of the 114 values were below the zero-time control). Non-detects were listed as the detection limit of 0.005 fg C aggregate^{-1} d^{-1} so they would plot on the log scale graph.

same 1 mm slice at 0.5 cm below surface; Fig. 3). More rapid rates and higher variability was also observed in the top 0.5 cm than further down core (Fig. 3).

Finally, the importance of sample size was more directly compared using two depths (1, 5 cm) of the duplicate corn field cores with one processed for the macroscale assay (Fig. 1) and those same depths selected from the data from the microscale assay (Fig. 3). Although the range was greater with the microaggregate assay, 93–2283 ng C g^{-1} d^{-1} at 1 cm and 23–150 ng C g^{-1} d^{-1} at 5 cm, than with the macroscale assay, 147–170 and 18–112 ng C g^{-1} d^{-1}, respectively, the means were quite comparable (Figs. 1 and 3). The mean (+/-SD) for one cm deep microaggregate sample was 423 (± 613) ng C g^{-1} d^{-1} versus 162 (± 13) ng C g^{-1} d^{-1} for the macroscale observation. For the five-cm depth, the microscale value was 63 ± 53 ng C g^{-1} d^{-1} versus 63 (± 47) ng C g^{-1} d^{-1} for the macroscale. This is some evidence that the process of subsampling microaggregates with the BioLP did not measurably inhibit bacterial production within the aggregate relative to the macroscale method.

4. Discussion

4.1. Bacterial production in soil

Over the past 30 years, the most accepted and commonly used measure of heterotrophic bacterial production of new aquatic biomass (also called secondary production) has involved the Leucine method (*e.g.,* Kirchman, 2001). In terrestrial ecosystems, leucine incorporation has been used since Bååth (1994), though somewhat less commonly than in aquatic systems. Aside from the direct evaluation of bacterial metabolism and use of dissolved organic matter, it has also been used in soil to measure toxicity (Demoling and Bååth, 2008), nutrient limitation (Demoling et al., 2007), effect of labile carbon addition and competition with fungi (Reischke et al., 2014). Using a BioLP to perform high resolution microscale sampling, we found rates of bacterial production to be on the same order as macroscale sampling performed on adjacent samples from the same core. This indicates that this BioLP sampling strategy produces data that is comparable in magnitude to that of the higher sample volume measurements. It is difficult to directly compare with those reported in the literature because the results are often presented as relative change in bacterial production of a treatment (*e.g.,* percent inhibition) rather than converted to rates of new bacterial biomass carbon (*i.e.,* μg C kg^{-1} d^{-1}) (Bååth et al., 2001; Demoling and Bååth, 2008, and references therein).

Also, amongst those literature values, there can be methodological differences that result in different rates. For instance, rewetting and incubation temperature can scale the rebound of bacterial production by an order of magnitude (Iovieno and Bååth, 2008; Pietikäinen et al., 2005). We resuspended the microaggregates in rainwater and used four-hr incubations which would have rates increasing to that similar to constantly wet soil (Iovieno and Bååth, 2008). A challenge with longer incubation times is the increased influence of bottle effects on the measurement (*e.g.,* Massana et al., 2001). Here, incubation time was chosen to balance assay sensitivity while minimizing likelihood of bottle effects.

Although the limit of detection can likely be improved, given that it could measure over two orders of magnitude differences amongst microaggregate groups, this level of sensitivity should be sufficient to establish relationships with other biogeochemical parameters. It is possible that further rinsing could lower this limit but it may be more useful to maintain the standard methodology (Smith and Azam, 1992) for biogeochemical comparison with literature values for heterotrophic bacterial production. The challenge is to be able to measure other such parameters at the microaggregate scale. Given the variability seen here with bacterial production on adjacent microaggregate groups, ideally the analyses would be made non-destructively on the same groups of microaggregates upon which bacterial production is measured.

4.2. Importance of microscale sampling

Typically biogeochemical parameters of soil are measured on a relatively large scale (> gram), in part, because of assay detection limits but also to obtain a more representative value for a parameter that can be extrapolated to the larger ecosystem (*e.g.,* corn field, forest). A consequence of this common approach is that values for these measurements have averaged out much of the important information associated with chemical or biological gradients that occur within the measurement scale (*e.g.,* Pedersen et al., 2015). By increasing the sample size, one influences the results until all patterns in the survey are lost (Ellingsøe and Johnsen, 2002; Kang and Mills, 2006). Macroscale soil sampling in this study (*ca.* 50 mg) involved about 5% or less of the mass typically used for measurement of bacterial production, yet we still found this same relationship between sample size and rate variability when compared with BioLP microaggregate sampling. Macroscale sampling did appear discreet enough to capture some variability at the interface between horizons (Fig. 1). Boundaries between distinct physical environments can often be an area of enhanced bacterial activity (Pinton et al., 2001) although maximal rates of bacterial production were much more rapid at shallower depths. Findings based on such large-scale measurements can be misleading or largely irrelevant to the process being investigated (Baveye and Laba, 2015).

4.3. Bacterial production of aggregates

Heterotrophic bacterial production of aggregates were first measured using relatively large (> 3 mm dia.) individual marine aggregates (*i.e.,* marine snow) with rates ranging from 7.7 to 331 ng C aggregate^{-1} d^{-1} (Alldredge et al., 1986). Aggregates collected from marine and freshwater systems were often pooled to reduce variability amongst production values for individual aggregates (Simon et al., 1990; Smith et al., 1992; Grossart and Simon, 1993, 1998). Heterotrophic production of individual aggregates was first measured in freshwater systems (*i.e.,* lake snow; 2–4 ng C aggregate d^{-1} for 5 mm aggregates) and was associated with elevated dissolved organic matter cycling (Grossart and Simon, 1993). Ploug and Grossart (1999) found that marine aggregates

(marine snow; 700 μm^3) had lower per aggregate bacterial production rates when measured in groups than when measured on individual aggregates (range: 4.6–576 ng C aggregate^{-1} d^{-1}). Both altered production and growth efficiency was attributed to reduced oxygen diffusion with collections of these larger aggregate (Grossart and Ploug, 2000) as is seen with bacterial microcolonies (Wessel et al., 2014). These rates were often more rapid than measured here in a soil system (maximum: 0.897 ng aggregate^{-1} d^{-1}; Fig. 3) though these soil microaggregates were typically much smaller than those used in the aquatic systems (ca. 300 μm vs 0.7–7 mm).

In soil systems, other bacterial assemblage parameters have been measured on macro- and microaggregate sized fractions of whole soil samples separated by sieving or thin-layer dissection with several studies having found distinct bacterial assemblage genomic differences amongst the different sized fractions or sections (*e.g.,* Blaud et al., 2014; Davinic et al., 2012; Vogel et al., 2003). Using relatively large aggregates (5–6 mm dia.), Rawlins et al. (2016) found six-fold magnitude variation in organic carbon mineralization rate which would be related to heterotrophic bacterial production rate. Our report here appears to be the first to measure rate of heterotrophic bacterial production on soil microaggregates.

4.4. 3D printing technology

BioLP 3D laser printing technology was used to subsample layers of soil into groups of adjacent microaggregates at a scale of resolution for live samples that was higher than other reported techniques. Previous methods for fine scale observations involve embedding the sample with resin prior to fine scale sectioning and thus cannot be used with live bacterial assemblages (*e.g.,* Nunan et al., 2001). Previous BioLP studies have provided evidence that this type of laser sampling was relatively nondestructive based on lack of bacterial heat shock protein induction and DNA damage amongst cultured strains (Ringeisen et al., 2004). Here we found that mean values for bacterial production were similar to that of parallel, higher-scale sampling which is also a line of evidence that the laser induced separation of microaggregates from higher level soil structure does not affect bacterial growth any more than more crude sampling strategies (*i.e.,* syringe coring). Any physical separation that breaks the soil structure would be expected to release low molecular weight dissolved organic matter from fungal hyphae or root rhizomes and this strategy does not address that effect on bacterial production. Its primary future use may be in evaluation of biogeochemical controls of heterotrophic bacterial production by enabling the correlation of chemical and physical traits of soil with bacterial growth at the resolution and microaggregate scale that is likely to be most important to its function in nature.

Though the BioLP printing strategy is very precise in geolocation within the sample adhered to the slide, the challenge is minimizing the disturbance and translocation of microaggregates in the transfer of the soil from nature to the BioLP slide. The ability to section a core slice is limited in one way by the maximum size of the particles present in the soil. Large inorganic grains at the section interface generally cannot be easily sliced and tend to get pushed or dragged through the more mobile phase of soil by the cutting device. Soils comprised mainly of finer grain particles tend to be easier to manipulate by most soil sectioning methods. Large organic aggregates can be sliced but also tend to compress prior to fracture and be dragged through the matrix. Methods used to hold the matrix in place prior to sectioning (*e.g.,* hardening with a resin) do not maintain viability of the microorganisms harbored within the microaggregates. In addition, most slicing strategies can spread microorganisms across the newly created interface of a section as the slicing device migrates across the soil to divide the core segments. This has the clear potential to cross contaminate adjacent sections of the core slice of the same depth with soil microorganisms contacted with the slicer from earlier in the core slicing process. Using the strategy implored here, cross contamination could occur among sections of the interface from the same depth but much less likely among sections of different depths. Specific to the BioLP constraints, subsections from a core slice (1 mm × 2 mm x 2 mm) had to be rewet and partially flattened to adhere to the inverted slide prior to printing which likely translocated adjacent microaggregates within that subsection.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2019.01.003.

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